Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification and crystallization of *Chaetomium thermophilum* Cu,Zn superoxide dismutase

Sachin Wakadkar,[a]‡ Li-Qing Zhang,[b,c]‡ Duo-Chuan Li,[b]* Teemu Haikarainen,[a] Prathusha Dhavala[a] and Anastassios C. Papageorgiou[a]*

[a]Turku Centre for Biotechnology, University of Turku and Åbo Akademi, Turku 20521, Finland, [b]Department of Environmental Biology, Shandong Agricultural University, Taian, Shandong 271018, People's Republic of China, and [c]Department of Chemistry and Chemical Engineering, Taishan Medical College, Taian, Shandong 271016, People's Republic of China

‡ These authors should be considered joint first authors.

Correspondence e-mail: lidc20@sdau.edu.cn, tassos.papageorgiou@btk.fi

Received 30 June 2010
Accepted 30 July 2010

Cu,Zn superoxide dismutase (Cu,ZnSOD) from the thermophilic fungus *Chaetomium thermophilum* was expressed in *Pichia pastoris* and purified. Crystals were grown in over 120 conditions but only those produced with 1.4 *M* sodium potassium phosphate pH 8.2 as precipitant were suitable for structural studies. Data were collected to 1.9 Å resolution at 100 K from a single crystal using a synchrotron-radiation source. The crystals belonged to space group $P6_1$/$P6_5$, with unit-cell parameters a = 90.2, c = 314.5 Å and eight molecules in the asymmetric unit. Elucidation of the crystal structure will provide insights into the active site of the enzyme and a better understanding of the structure–activity relationship, assembly and thermal stability of Cu,ZnSODs.

1. Introduction

Superoxide dismutases (SODs; EC 1.15.1.1) are a family of metalloenzymes that catalyze the dismutation of superoxide radicals ($O_2^{\bullet-}$) to H_2O_2 and O_2, thereby protecting cells against the harmful effects of free superoxide radicals (Imlay, 2008). The latter can damage all types of biomolecules, including DNA, proteins and membrane lipids, and can lead to mutagenesis, inhibition of growth and cell death (Bannister *et al.*, 1987; Hassan, 1989; Fridovich, 1995).

Four main types of SODs have been identified based on their active-site metal requirements: copper–zinc SODs (Cu,ZnSODs), manganese SODs (MnSODs), iron SODs (FeSODs) and nickel SODs (NiSODs) (Abreu & Cabelli, 2010). Cu,ZnSODs are not related to the other types of SOD (Miller, 2004; Wintjens *et al.*, 2004; Perry *et al.*, 2010) and operate as homodimeric enzymes of approximately 2 × 16 kDa. Each subunit contains an active site which contains one copper and one zinc cation that are bridged by a histidine imidazole. Three additional histidines complete the copper ligation, resulting in an overall distorted square-planar structure. Crystallographic studies have shown a conserved architecture characterized by a flattened antiparallel β-barrel composed of eight strands that are arranged in a Greek-key topology. Mutations in human Cu,ZnSOD can result in structural destabilization of the dimer assembly, leading to fibril formation. Such fibrils have been found to be responsible for motor neuron death in about 20% of cases of familial amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease (DiDonato *et al.*, 2003).

Recombinant SODs have been proposed to be clinically useful for a wide variety of disorders, including prevention of oncogenesis, tumour promotion and tumour invasiveness and reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Trotti, 1997; Matés & Sánchez-Jiménez, 2000; Angelova *et al.*, 2001; Zwacka *et al.*, 1998). However, recombinant Cu,ZnSOD is rapidly cleared from human serum and shows a short biological half-life of 20 min in healthy volunteers (Kaipel *et al.*, 2008). Understanding the stability of Cu,ZnSOD is therefore important for the creation of new enzymes with better properties for use in biomedical applications. In this regard, eukaryotic thermophilic organisms have been suggested as a better source of enzymes that are highly similar to those from humans (Shin *et al.*, 2009).

In recent years, there has been increasing interest in thermophilic fungi as a source of thermostable enzymes for use in industrial and biomedical applications. Although a number of thermostable cellulases, proteases, amylases and xylanases have been reported from thermophilic fungi (Maheshwari *et al.*, 2000), to date only one study of a Cu,ZnSOD from a thermophilic fungus, that from *Thermoascus aurantiacus* var. *levisporus*, has been reported (E *et al.*, 2007). Here, we describe the expression, purification and crystallization of a Cu,ZnSOD (CZ1) from *Chaetomium thermophilum*, a soil-borne thermophilic fungus that grows well at temperatures around 323 K. This is the first crystallization report of a thermostable fungal Cu,ZnSOD (with a sequence identity of ∼56% to the human SOD) and the determination of its crystal structure will provide a better understanding of thermostability issues in SODs.

2. Materials and methods

2.1. Expression and purification

2.1.1. Construction of the expression plasmid. Previous attempts to express the enzyme in *Escherichia coli* strain BL21 using the pEHISTEV vector resulted in low yield and stability. Moreover, removal of the His_6 tag from the purified enzyme by TEV protease caused loss of the protein. Thus, the yeast expression system was chosen in this study as an alternative. Based on the *C. thermophilum* Cu,ZnSOD gene sequence (EMBL accession No. DQ493760; *cz1*), a pair of specific primers containing restriction sites (BS1, 5′-CCGAATTCGTCAAGGCAGTTGCTGT-3′, with an *Eco*RI recognition site, and BX1, 5′-GGGCGGCCGCTTACTGGGCAATGCCAAT-3′, with a *Not*I recognition site) were designed to amplify the complementary DNA (cDNA) sequence encoding mature CZ1. The PCR product was digested with restriction enzymes and subcloned into the expression vector pPIC9K (Invitrogen). The positive recombinants were confirmed by PCR amplification with a pair of primers corresponding to the 5′AOX1 and 3′AOX1 sequencing primers (Invitrogen). The resulting recombinant plasmid was designated pPIC9K/cz1 and was subsequently confirmed by PCR analysis, restriction analysis and DNA sequencing.

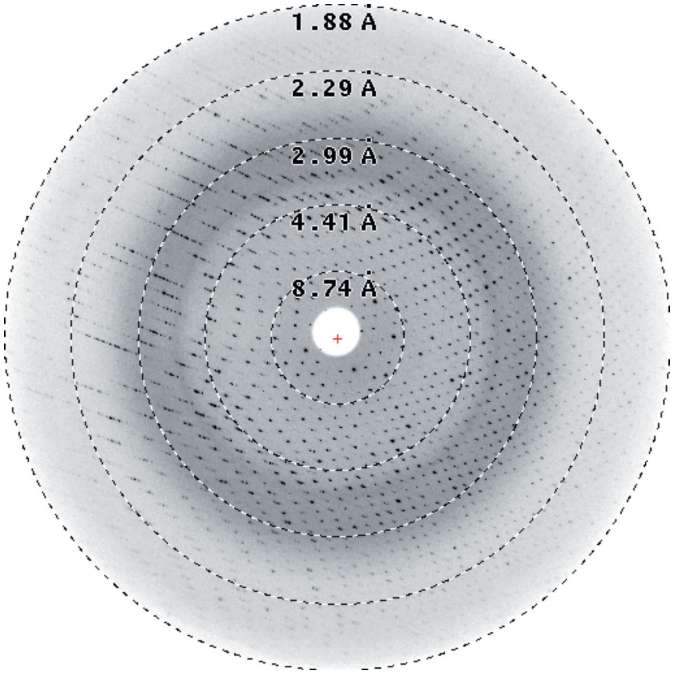


Figure 1
A typical diffraction image of the CZ1 crystals used for data collection. The resolution rings are shown.

2.1.2. Transformation of *P. pastoris* GS115 and screening of transformants. The *Sac*I-linearized recombinant plasmid pPIC9K/cz1 was transformed into *P. pastoris* GS115 by electroporation with an Eppendorf Electroporator 2510. For verification of the gene integrated into the *P. pastoris* genome, genomic DNA of a number of transformants was isolated. The copy number of the gene integration in the genome of *Pichia pastoris* was determined to be between 7 and 12. PCR amplifications were carried out based on genomic DNA using primers corresponding to α-factor and 3′AOX1 sequencing primers (Invitrogen).

2.1.3. Induced expression in *P. pastoris*. The transformants were grown in BMGY at 301 K until the A_{600} reached ∼1.5. The cells were harvested by centrifugation at 5000*g* for 5 min and then cultured in BMMY. Expression was induced by supplementing the BMMY with 1% methanol; a second sample with no methanol added was used as a control. Aliquots of each culture were removed at 24 h intervals and the induced expression of the recombinant CZ1 was detected by SDS–PAGE.

2.1.4. Purification of CZ1. Transformed yeast was cultured at 301 K for 7 d in BMMY according to the *Pichia* Expression System Kit (Invitrogen). The culture filtrate was centrifuged at 10 000*g* for 10 min at 277 K and the resultant supernatant was dialysed overnight against three changes of 50 m*M* Tris–HCl pH 8.0 (buffer *A*). The dialysed sample was loaded onto a DEAE-Sepharose column (1 × 20 cm) equilibrated with buffer *A*. After the column had been washed with five column volumes of buffer *A*, a 240 ml linear gradient of NaCl (0–0.3 *M* in buffer *A*) was applied at a flow rate of 60 ml h^{-1}. The volume of each fraction was 3 ml. CZ1 activity was determined using the method described by Stewart & Bewley (1980) and active fractions were collected. The purity of the recombinant CZ1 was assessed by SDS–PAGE. The protein concentration was determined by the method of Bradford (1976) using crystalline bovine serum albumin as the standard.

2.2. Crystallization and data collection

The purified enzyme was concentrated by ultrafiltration using Amicon Ultra Centrifugal Filters (10 000 molecular-weight cutoff; Millipore) and stored in 20 m*M* Tris–HCl pH 8.2 after buffer exchange. The final protein concentration was 10.5 mg ml^{-1} as measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific). Initial crystallization trials were performed manually with Index (Hampton Research), PACT (Qiagen) and SaltRx (Hampton Research) crystal screens using the sitting-drop vapour-diffusion method at 289 K in 96-well Corning plates. The drops contained 0.75 µl protein solution and an equal volume of precipitant solution. Crystals were found after 2 d in 71, 51 and 1 conditions of PACT, Index and SaltRx, respectively. All conditions in PACT and Index that produced crystals contained PEG as the main precipitant. For optimization, nine conditions from PACT, eight conditions from Index and the single condition from SaltRx were selected. These conditions were chosen based on the crystal size and shape and the relative time of crystal growth. Optimization of the crystallization conditions for different salt concentrations, pH values and PEG concentrations was performed using the hanging-drop vapour-diffusion method. Crystals were found in all optimized conditions. Initial screening for X-ray diffraction using an in-house X-ray generator (Rigaku RU-200B equipped with a MAR 345 imaging-plate detector) showed that only the crystals produced using the SaltRx condition exhibited a good

Table 1
Crystal parameters and data-processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P6_1/P6_5$
Unit-cell parameters (Å)	a = 90.2, c = 314.5
Resolution range (Å)	20–1.9 (2.0–1.9)
Wavelength (Å)	0.8123
Temperature (K)	100
Total reflections	358237 (49713)
Unique reflections	59749 (8417)
Completeness (%)	98.1 (99.1)
Mosaic spread (°)	0.6
$R_{\rm merge}$† (%)	7.4 (37.0)
$R_{\rm meas}$‡ (%)	8.1 (40.6)
$\langle I/\sigma(I)\rangle$	19.1 (5.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl. ‡ The redundancy-independent R value, calculated as $R_{\rm meas} = \sum_{hkl}[N/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where N denotes the redundancy (Diederichs & Karplus, 1997).

diffraction pattern; the rest all showed the presence of two or more crystal lattices that were inseparable. Thus, crystals suitable for X-ray crystallographic analysis were subsequently grown at 289 K by mixing 2.5 µl protein stock solution with an equal volume of a reservoir solution consisting of 1.4 *M* sodium potassium phosphate pH 8.2. The hanging drops were equilibrated against 800 µl reservoir solution and crystals grew to their final size in about 4 d. Data were collected to 1.9 Å resolution on the X13 beamline at EMBL Hamburg (c/o DESY) from a single crystal that had been soaked for a few seconds in crystallization solution containing 25%(*v*/*v*) glycerol as a cryoprotectant and flash-cooled to 100 K in a nitrogen-gas stream (Fig. 1). A total of 150 images were recorded using a MAR CCD detector with 0.5° rotation per image, a wavelength of 0.8123 Å, a crystal-to-detector distance of 174 mm and an average exposure time of 10–15 s per image in constant count (dose) mode to compensate for the decay of the X-ray beam intensity during data collection. Data were processed with the *XDS* package (Kabsch, 2010) and final statistics are summarized in Table 1.

3. Results and discussion

3.1. Expression and purification

The *C. thermophilum cz1* gene was successfully overexpressed in *P. pastoris* GS115 when induced by methanol supplementation. The highest activity was obtained after 5 d of induction and the expression level was ∼2.6 mg ml^{-1}. The expressed protein was purified from the culture filtrate by a single ion-exchange chromatography step. The purified enzyme showed an activity of about 127.4 U mg^{-1}. Electrophoresis of the recombinant CZ1 on SDS–PAGE gave a single band with a molecular mass of about 17.7 kDa (Fig. 2). This value is higher than the value of 16.1 kDa estimated from the deduced amino-acid sequence, suggesting that the recombinant CZ1 might be glycosylated. The availability of pure enzyme will allow a full biochemical and structural characterization, including assessment of its thermal stability. Preliminary studies of CZ1 enzyme activity showed an optimal temperature of about 333 K at pH 6.5. Moreover, the enzyme retained approximately 93% of its activity after incubation at 333 K for 60 min.

3.2. Crystallization of CZ1

Although crystals of CZ1 were produced in numerous conditions (Fig. 3), diffraction-quality crystals were only obtained in one condition that contained sodium potassium phosphate as precipitant and no PEG. All crystals grown in the presence of PEG exhibited multiple lattices. The crystals of CZ1 used for data collection belonged to space group $P6_1/P6_5$, with unit-cell parameters a = 90.2, c = 314.5 Å.

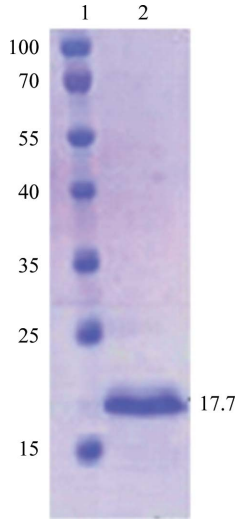
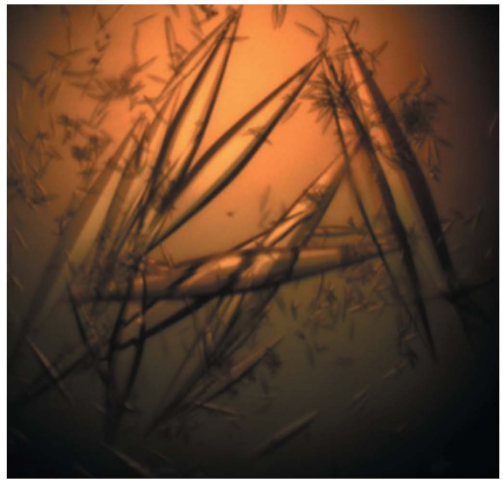


Figure 2
Purity of CZ1 on 12% SDS–PAGE. Lane 1, molecular-weight protein markers (kDa); lane 2, purified CZ1 visualized by Coomassie Brilliant Blue staining.

(*a*)



(*b*)

Figure 3
Crystals of CZ1. (*a*) Representative crystals obtained using PEG as precipitant. (*b*) Crystals obtained with sodium potassium phosphate. These crystals have typical dimensions of 0.6 × 0.15 × 0.15 mm.

Assuming the presence of eight molecules of the enzyme in the asymmetric unit and a molecular weight of 16 100 Da, the Matthews coefficient V_M (Matthews, 1968) is 2.56 Å^3 Da^{-1}, corresponding to a solvent content of ∼52%. Structure determination will be pursued by the molecular-replacement method using, for example, the structure of yeast Cu,ZnSOD (PDB code 1f1g; sequence identity of ∼70%; P. J. Hart, N. L. Ogihara, H. Liu, A. M. Nersissian, J. S. Valentine & D. Eisenberg, unpublished work) in the first instance.

We thank the Academy of Finland (Grant 121278 to ACP), the Finnish National Informational and Structural Biology Graduate School, the Chinese National Nature Science Foundation, the Chinese National Programs for High Technology Research and Development and the Chinese Project of Transgenic Organisms for financial support. Access to EMBL Hamburg c/o DESY was provided by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement No. 226716.

References

Abreu, I. A. & Cabelli, D. E. (2010). *Biochim. Biophys. Acta*, **1804**, 263–274.
Angelova, M., Dolashka-Angelova, P., Ivanova, E., Serkedjieva, J., Slokoska, L., Pashova, S., Toshkova, R., Vassilev, S., Simeonov, I., Hartmann, H. J., Stoeva, S., Weser, U. & Voelter, W. (2001). *Microbiology*, **147**, 1641–1650.
Bannister, J., Bannister, W. & Rotilio, G. (1987). *CRC Crit. Rev. Biochem.* **22**, 111–180.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
DiDonato, M., Craig, L., Huff, M. E., Thayer, M. M., Cardoso, R. M., Kassmann, C. J., Lo, T. P., Bruns, C. K., Powers, E. T., Kelly, J. W., Getzoff, E. D. & Tainer, J. A. (2003). *J. Mol. Biol.* **332**, 601–615.
Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Biol.* **4**, 269–275.
E, S., Guo, F., Liu, S., Chen, J., Wang, Y. & Li, D. (2007). *Biosci. Biotechnol. Biochem.* **71**, 1090–1093.
Fridovich, I. (1995). *Annu. Rev. Biochem.* **64**, 97–112.
Hassan, H. M. (1989). *Adv. Genet.* **26**, 65–97.
Imlay, J. A. (2008). *Annu. Rev. Biochem.* **77**, 755–776.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 125–132.
Kaipel, M., Wagner, A., Wassermann, E., Vorauer-Uhl, K., Kellner, R., Redl, H., Katinger, H. & Ullrich, R. (2008). *J. Aerosol. Med. Pulm. Drug Deliv.* **21**, 281–290.
Maheshwari, R., Bharadwaj, G. & Bhat, M. K. (2000). *Microbiol. Mol. Biol. Rev.* **64**, 461–488.
Matés, J. M. & Sánchez-Jiménez, F. M. (2000). *Int. J. Biochem. Cell Biol.* **32**, 157–170.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Miller, A. F. (2004). *Curr. Opin. Chem. Biol.* **8**, 162–168.
Perry, J. J., Shin, D. S., Getzoff, E. D. & Tainer, J. A. (2010). *Biochim. Biophys. Acta*, **1804**, 245–262.
Shin, D. S., Didonato, M., Barondeau, D. P., Hura, G. L., Hitomi, C., Berglund, J. A., Getzoff, E. D., Cary, S. C. & Tainer, J. A. (2009). *J. Mol. Biol.* **385**, 1534–1555.
Stewart, R. & Bewley, J. (1980). *Plant Physiol.* **65**, 245–248.
Trotti, A. (1997). *Curr. Opin. Oncol.* **9**, 569–578.
Wintjens, R., Noël, C., May, A. C., Gerbod, D., Dufernez, F., Capron, M., Viscogliosi, E. & Rooman, M. (2004). *J. Biol. Chem.* **279**, 9248–9254.
Zwacka, R. M., Zhou, W., Zhang, Y., Darby, C. J., Dudus, L., Halldorson, J., Oberley, L. & Engelhardt, J. F. (1998). *Nature Med.* **4**, 698–704.